The Journal of
GENE MEDICINE

Volume 4 Number 5 September–October 2002

Contents

...l 4 No. 6 pp. 579–698

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The Journal of Gene Medicine

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Aims and Scope

The Journal of Gene Medicine is a print and electronic journal which publishes articles on the science of gene transfer and its clinical applications. The journal will consider articles on all aspects of gene therapy including design and production of vectors, research into the mechanisms underlying gene transfer, preclinical studies including animal models, developmental aspects (large-scale production, toxicology) and clinical trials. The editors particularly welcome articles dealing with the methodological aspects of gene transfer *in vivo*, notably in the context of human studies. Articles addressing more fundamental biological issues which could open up avenues for more effective gene transfer are also welcome.

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The Journal of Gene Medicine

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The Journal of Gene Medicine (Print ISSN 1099-498X; Online ISSN 1521-2254 at Wiley InterScience, www.interscience.wiley.com) is published bi-monthly by John Wiley & Sons, Ltd., The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, UK.

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Volume 4 2002 6 issues
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Production Information

Production Details

Production Editor
Freyja Tipping
E-mail: ftipping@wiley.co.uk

Printed on acid-free paper.
Typeset by Laserwords Private Ltd, Chennai, India.
Printed and bound in the United Kingdom by Henry Ling Limited, at the Dorset Press, Dorchester, DT1 1HD.

Identification Statement

The Journal of Gene Medicine (ISSN 1099-498X, USPS 018-587) is published bi-monthly by John Wiley & Sons, Ltd., The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, UK. Periodicals postage paid at Rahway, NJ. Air freight and mailing in the USA by Mercury Airfreight International Ltd Inc., 365 Blair Road, Avenel, NJ 07001, USA.
USA POSTMASTER – send address changes to *The Journal of Gene Medicine*, c/o Mercury Airfreight International Ltd Inc., 365 Blair Road, Avenel, NJ 07001, USA.

The Journal
GENE MEDIC

In this iss

Gene therapy for rheumatoid arthritis : a review

Rheumatoid arthritis (RA) is a severe systemic autoimmune disease in which chronic synovial inflammation results in destruction of the joints. There is no truly effective treatment for RA. Bessis *et al.* review the current status of a gene therapy approach for the treatment of the disease. Potential strategies include down-regulating mediators of inflammation or articular destruction (such as TNF-α or IL-1) and up-regulating anti-inflammatory cytokines (such as IL-4 and IL-10). The authors also review the gene delivery systems that have been used and they consider local versus systemic, and *in vivo* versus *ex-vivo* strategies. *Ex vivo* gene transfer has been investigated using synovial cells, fibroblasts, T cells, dendritic cells, and various xenogeneic cells. Clinical trials have started with retroviruses (*ex vivo*) expressing the IL-1 receptor antagonist and have demonstrated the feasibility of the strategy. The best target molecules remain to be determined and extensive pre-clinical studies will need to be performed. (*p. 581*)

Cell surface protease targeting of a cytotoxic GALV Env?

Kirkham *et al.* investigated the possibility of targeting the cytotoxic activity of a hyperfusogenic Gibbon Ape Leukaemia virus (GALV) envelope glycoprotein therapeutic gene whilst simultaneously enhancing its immune stimulatory properties via local, matrix-metalloprotease (MMP)-mediated release of human GM-CSF. Hyperfusogenic GALV envelopes, whose expression is known to be highly cytotoxic, were fused at the N-terminus to 'blocking' via MMP-sensitive linkers trol linkers (non-cleavable Xa protease-cleavable link their cytotoxicity was asse MMP positive and negative Unlike protease targeting in text of retroviral vectors, activation of the cytotoxicity envelope by cleavage of blocking ligand did not app specifically mediated by cel MMPs. Thus, it appears th ficity of cell-cell fusion med GALV envelope cannot be lated in the same fashion as fusion. (*p. 592*)

Optimizing retroviral transduction of murine

Gene transfer using retrovir duction offers the advan long-term transgene expressi developing strategies that dritic cells (DC) for imm apy. The goal of this st to define optimal condition transduction of murine bo row (BM)-derived DCs. Fres report here that protamin and IL-4 allow to increase D ral transduction, whereas p induced DC apoptosis. DC ated in GM-CSF plus IL-4 p however a more mature ph These findings have potenti cations in experimental gene (*p. 601*)

Retina gene therapy in VI cats

Ho *et al.* successfully used a associated virus (AAV) to retinal manifestations of th mal storage disease, muco charidosis VI (MPS VI), i

THE JOURNAL OF GENE MEDICINE
J Gene Med 2002; 4: 644–654.
Published online 13 August 2002 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/jgm.295

RESEARCH ARTICLE

Improved antisense oligonucleotide induced exon skipping in the *mdx* mouse model of muscular dystrophy

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Received: 3 January 2002
Revised: 15 April 2002
Accepted: 18 April 2002

Abstract

Background Duchenne muscular dystrophy (DMD) is a fatal genetic disorder caused by dystrophin gene mutations that preclude synthesis of a functional protein. One potential treatment of the disorder has utilised antisense oligoribonucleotides (AOs) to induce removal of disease-associated exons during pre-mRNA processing. Induced in-frame mRNA transcripts encode a shorter but functional dystrophin. We have investigated and improved the design of AOs capable of removing exon 23, and thus the disease-causing nonsense mutation, from mRNA in the *mdx* mouse model of DMD.

Methods H-2K^{b}-*tsA58 mdx* cultures were transfected with complexes of Lipofectin and AOs. Exon skipping was detected by RT-PCR and subsequent protein production was demonstrated by Western blotting. AOs were delivered at a range of doses in order to compare relative efficiencies.

Results We describe effective and reproducible exon 23 skipping with several AOs, including one as small as 17 nucleotides. Furthermore, the location of a sensitive exon 23 target site has been refined, whilst minimum effective doses have been estimated *in vitro*. These doses are significantly lower than previously reported and were associated with the synthesis of dystrophin protein *in vitro*.

Conclusions These results demonstrate the increasing feasibility of an AO-based therapy for treatment of DMD. By refining AO design we have been able to reduce the size and the effective dose of the AOs and have dramatically improved the efficiency of the technique. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords antisense oligonucleotides; dystrophin; exon skipping; Duchenne muscular dystrophy

Introduction

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular condition resulting from an absence of dystrophin protein due to either nonsense or frame-shift mutations in the dystrophin gene [1]. In the absence of genetic screening, DMD has an incidence of 1 in 3500 live male births, with 1 in 3 cases resulting from a *de novo* mutation. Dystrophin-negative muscle fibres are weaker and undergo repetitive cycles of damage and repair following muscle contraction. Cycles of degeneration and regeneration are eventually

exhausted and the muscle is gradually replaced by adipose and connective tissue until patients die from respiratory or cardiac failure, usually before the third decade of life [2]. A milder allelic form of the disease, called Becker muscular dystrophy (BMD), is associated with a range of phenotypes ranging from mild to severe (borderline DMD). BMD dystrophin mutations typically give rise to shortened, in-frame transcripts associated with a dystrophin protein of reduced quantity or quality [3].

Gene therapy strategies for the treatment of DMD have so far been met with disappointment. Gene replacement has been hindered by host immune responses to first-generation viral vectors [4]; naked plasmid DNA transfer by direct intramuscular injection is inefficient [5]; myoblast transfer and stem cell therapy have respectively fought to overcome poor survival of transplanted cells [6] and very limited cell recruitment [7]; homologous gene (utrophin) upregulation may not colocalise neuronal nitric oxide synthase (nNOS) [8] or compensate for the absence of the various isoforms of dystrophin [9]; gene repair strategies mediated by chimeric oligonucleotides [10] or short fragment homologous recombination [11] are inefficient to date but offer potential for *ex vivo* correction of host myoblasts; and aminoglycoside therapy [12] is only applicable to a small subset of (nonsense) mutations that cause the disease.

Recent work by our group [13] and others [14] has revealed the potential for an alternative strategy for treating DMD utilising antisense oligoribonucleotides (AOs) to induce targeted removal of disease-causing exons from pre-mRNA transcripts during splicing. Unlike most other antisense applications which aim to destroy specific RNA targets, such as targeting oncogenes in cancer therapy [15], producing viable dystrophin mRNA capable of being translated into a semi-functional protein is mandatory. For the application of AOs to DMD to be successful, the chemistry of the AOs must be such that targeted degradation of dystrophin mRNA is avoided. Inclusion of a phosphorothioate (PS) backbone retains the anionic charge and increases resistance to nucleases. More importantly, methylation of the 2′ oxygen generates an RNA-like molecule that will evade RNase H mediated degradation of the target induced by DNA:RNA hybrids [16].

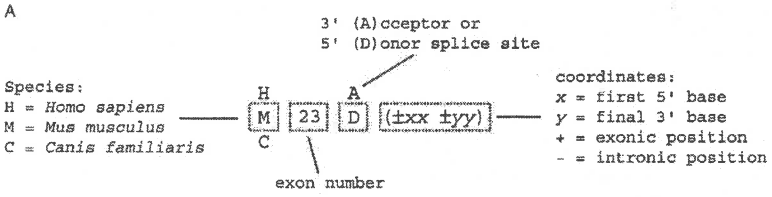
Inducing successful exon skipping requires delivering 2′-*O*-methylated PS AOs to the nucleus of muscle cells and their hybridisation to sequence motifs involved in splicing. Blocking these sequences interferes with spliceosome assembly, thereby redirecting the splicing process into excluding the undesirable exon and the flanking introns from the mature mRNA transcript. AO-modified splicing with therapeutic potential has been reported for the dystrophin pre-mRNA [13,14,17–19], for the β-globin transcripts in thalassemia [20], in survival motor neuron 2 (SMN2) pre-mRNA [21], as well as a cryptic splice site mutation in the *cystic fibrosis transmembrane conductor regulator* (CFTR) gene amongst others [22]. As ~15% of point mutations that cause genetic disease affect pre-mRNA splicing, it is probable that the number of diseases/mutations capable of being treated with

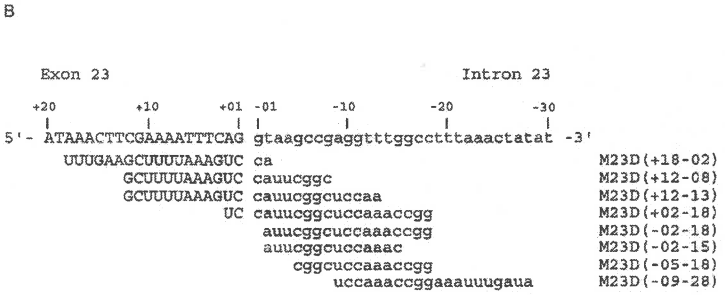
by Western blotting. We show here that an AO as small as 17 nucleotides can induce strong and consistent exon skipping and subsequent dystrophin protein synthesis. One AO could induce dystrophin protein at a dose as low as 5 nM. This dose is orders of magnitude lower than reported to cause exon skipping in our laboratory [13] and elsewhere [14,17].

Materials and methods

AO design

We have adopted a nomenclature for naming AOs targeted against dystrophin pre-mRNA that provides information about species specificity, the target site relative to the target exon and the exact annealing position (and thereby the AO size). Each AO name can be divided into two parts, a descriptive component and an exact coordinate position, as summarised in Figure 1A. A more detailed explanation of the nomenclature can be found on the Internet [31]. All AOs were 2′-*O*-methylphosphorothioate molecules synthesised and HPLC-purified by Geneworks (Adelaide, Australia). The sequence for intron 23 (Genbank accession AF062380) was used to design the sequences, which are shown schematically in Figure 1B. Several of the AOs have been described previously [13,19] and have been renamed here according to the nomenclature.





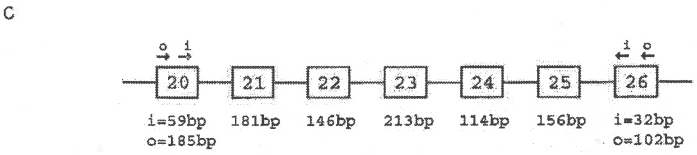


Figure 1. Nomenclature, sequence and location of antisense oligonucleotides and PCR primers. (A) Proposed nomenclature for the naming of AOs targeted against dystrophin pre-mRNA is divided into four parts: species, exon, (D)onor or (A)cceptor splice site and coordinates of the target site relative to the pre-mRNA sequence. (B) Sequences and schematic alignments of the AOs targeted against the donor splice site of intron 23 of mouse dystrophin with details of the numbering employed to assign the AO coordinates. Exonic bases are in upper case and are assigned positive (+) numbers; intronic bases are in lower case and assigned negative (−) numbers. M23D(+12-08) was previously named AO 5′SS-20 [19]; M23D(+12-13) was previously named AO 5′SS-25 [13]. All other AOs have not been previously reported. Sense and scrambled AOs designed as controls for M23D(+02-18) were also utilised and had the following sequences (5′ → 3′) AGGUAAGCCGAGGUUGGCC and CCUAUCGGCUCACAACCGUG, respectively. (C) Relative location of PCR primers and exon sizes for prediction of induced products: exons are represented by boxes and introns by lines (not to scale). Exon sizes in base pairs are indicated. Inner (i) and outer (o) nested PCR primers amplify only a portion of exon 20 or 26, which are also indicated. The sequence of PCR primers has been reported previously [13]

Cell culture and transfection

H-2K^b-*tsA58* (H-2K) normal and *mdx* cells were cultured exactly as described previously [13]. Transfections were carried out as follows. Complexes of Lipofectin (Life Technologies, Melbourne, Australia) and AO were always prepared in a 2:1 Lipofectin/AO ratio (w/w) in serum-free Opti-MEM (Life Technologies) according to the manufacturer's instructions, and, unless specified, transfections utilised 1 μg of AO (~300 nM). For the titration experiments (Figure 3), the amount of AO and consequently Lipofectin was varied according to the dose, although the ratio was always maintained at 2:1 (w/w). In all experiments, cells were exposed to AO/Lipofectin complexes for 3 h in serum-free Opti-MEM, after which the media was replaced with DMEM supplemented with 5% horse serum. For the standard transfections and titrations (Figures 2 and 3, respectively), H-2K *mdx* cells were transfected 48 h after plating in a total volume of 0.5 ml at a density of 2×10^4 cells/well in 24-well plates. Cells were transfected as duplicate wells and extracted RNA was pooled. For RNA time-course experiments (Figure 4), 1×10^5 cells were transfected 24 h after plating in 35-mm dishes. The transfection volume was 2 ml and RNA was extracted from individual dishes every second day after transfection (not plating).

RNA extraction, RT-PCR and S1 nuclease digestion of heteroduplexes

RNA was extracted from H-2K *mdx* cells with RNAzol B (Tel-Test, Friendswood, Texas) 24 h after transfection exactly as described previously [13]. RT-PCR was also performed as described, except the number of cycles in the primary and secondary (nested) amplification steps was reduced from 40 to 30 and from 30 to 25 cycles,

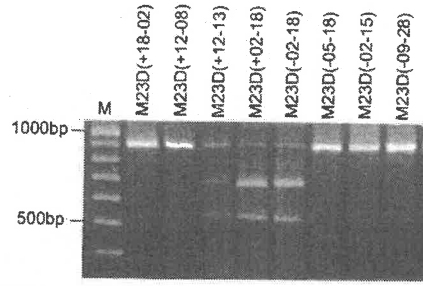


Figure 2. Detection of AO-induced dystrophin mRNAs skipping exon 23 by r
transfected with 1 μg of the respective AO complexed with Lipofectin as des
exons 20 and 26. Full-length unskipped (901 bp) mRNA was amplified from al
Smaller products corresponding to exon 23 (688 bp) and exon 22 and 23 remo
cells transfected with M23D(+02-18), M23D(−02-18) and M23D(+12-13). No
exon skipping. The gel image represents the PCR products after S1 nuclease d

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